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Effect of β-carotene on cell proliferation and differentiation of adipose-derived stem cells into endothelial progenitor cells

Wahyu Widowati[1], Caroline Tan Sardjono[1], Laura Wijaya[2], Dian Ratih Laksmitawati[3], Jeanne Adiwinata Pawitan[4]*, Ferry Sandra[5]

[1]Medical Research Center, Faculty of Medicine, Maranatha Christian University, Bandung, (INDONESIA)

[2]Stem Cell and Cancer Institute, Jakarta, (INDONESIA)

[3]Faculty of Pharmacy, Pancasila University, Jakarta, (INDONESIA)

[4]Department of Histology, Faculty of Medicine, Universitas Indonesia, Jakarta, (INDONESIA)

[5]Department of Biochemistry and Molecular Biology, Faculty of Dentistry, Trisakti University, Jakarta, (INDONESIA)

E-mail: jeanneadiwip@fk.ui.ac.id; jeanneadiwip@gmail.com

ABSTRACT

Aim: To evaluate the ability of β-carotene to protect oxidation effect of H_2O_2, and to enhance proliferation and differentiation into AD-MSCs into EPCs. **Experimental:** AD-MSCs were induced by EGM Bullet Kit medium with or without β-carotene. The proliferation of EPCs was determined by viable cell number counts. Differentiation into EPCs was characterized by the following surface markers: CD 34, CD133 and vascular endothelial growth factor receptor 2 (VEGFR-2). The protective effect of β-carotene was measured base on the level of intracellular reactive oxygen species (ROS) by fluorescence with 2',7'- dichlorofluorescein diacetate (DCF-DA) using flow cytometry. **Results:** β-Carotene enhanced cell proliferation and differentiation of AD-MSCs into EPCs, and also decreased the accumulation of H_20_2-induced intracellular ROS in EPCs. **Conclusion:** β-Carotene plays a role in cell proliferation and differentiation of AD-MSCs to EPCs that might be due to decreased intracellular ROS level.

KEYWORDS

Endothelial progenitor cell;
Oxidative stress;
Reactive oxygen species (ROS);
β-carotene;
Antioxidant;
Endothelium.

INTRODUCTION

Mesenchymal stem cells (MSCs) appear to be both multipotent and immune privileged, which make them particularly attractive for stem cell therapy[1,2]. Mesenchymal stem cells have improved heart function in both animal models of acute myocardial injury as well as clinical studies of patients with heart failure[1,3]. The mechanism of improvement involved their differentiation into smooth muscle and endothelial cells, which caused neovascularization and improved cardiac function[4]. Cell population, termed processed lipoaspirate (PLA) cells, can be isolated from human lipoaspirate, and can differentiate into the osteogenic, adipogenic, myogenic, and

chondrogenic lineages. Processed lipoaspirate cells expressed multiple CD marker antigens similar on MSCs[5].

Endothelial dysfunction plays a major role in the development and clinical complications of heart failure. Endothelial progenitor cells (EPCs) have been shown to provide an endogenous repair mechanism to counteract detrimental risk factor-induced effects and replace dysfunctional endothelium. Enhancing the number and function of EPCs with targeted interventions may elicit functional improvement in individuals with heart failure due to cardiovascular disease (CVD)[6].

Free radical formation is associated with the normal natural metabolism of aerobic cells[7]. The resulting reactive oxygen species (ROS) serve as secondary intracellular messengers and affect the overall redox status of a cell. The intracellular redox environment has a critical role in controlling apoptosis, proliferation, self-renewal, senescence, and differentiation. Dysregulation of any of these processes in EPCs will alter endothelial cell (EC) function, predisposing to the development of vascular pathology[8]. To contribute to tissue repair, EPCs and stem cells have to be equipped with anti-oxidative defense system to survive[9].

In cohort studies, high intake of β-carotene is associated with a lower incidence of and mortality from CVD[10]. Moreover, epidemiological studies have shown that a high intake of β-carotene is associated with a decreased risk for coronary artery disease[11]. Dietary intake of β-carotene or high level β-carotene in serum and adipose tissue shows inverse associations with CVD[12]. Beta carotene is a lipid-soluble antioxidant that rapidly scavenges reactive oxygen intermediates[11], and ROS[13], which protects LDL from oxidation[14]. Antioxidants also counterbalance the production of ROS that may cause oxidative damage to cells and modify cell growth regulatory pathways[15-17]. Further, previous studies suggested the role of β-carotene in the regulation of endothelium differentiation[18].

Therefore, the aim of this study is to evaluate the ability of β-carotene to enhance cell proliferation of AD-MSCs and their differentiation into EPCs. In addition, to evaluate β-carotene protection effect on H_2O_2 oxidation in AD-MSCs.

EXPERIMENTAL

This is a descriptive experimental study that was done in the Stem Cell and Cancer Institute, Jakarta, from July 2010 through December 2010. All protocols were reviewed and approved by the Stem Cell and Cancer Institute Institutional Review Board prior to the study.

MSC isolation from lipoaspirates

Lipoaspirates were obtained with informed consent from individuals undergoing tumescent liposuction surgery. Lipoaspirates were stored at 2–8°C for no longer than 24 hours. Methods used to isolate the MSCs from lipoaspirate were adopted from Sardjono *et al*. (2009)[19].

The raw lipoaspirates (120ml) were diluted with equal volume of Phosphate Buffered Saline (PBS) and divided in 50ml-tubes. The diluted lipoaspirates were centrifuged at 430×g for 10 minutes continuously at 20°C. After centrifugation, the target cell–containing lipid phase was removed from the top and transferred into new tubes and diluted with an equal volume of PBS. This washing step was repeated twice followed by further an equal volume dilution of cell-containing lipid fraction with pre-warmed (37°C) 0.075% collagenase type I (Sigma C-9722) in PBS. Enzyme digestion was done by incubation at 37 °C for 30 minutes on an orbital shaker. After digestion, enzyme activity was neutralized by adding equal volume of 10% fetal bovine serum (FBS [Invitrogen 26140]) containing DMEM (Gibco 11965-092). Digested product was then subjected to centrifugation at 600×g for 10 minutes. Pellet was re-suspended in DMEM with 10% PBS, and filtered through a 100-μm strainer mesh that was attached to a vacuum-pump to remove cellular debris. Collected cells after filtration were then ready for culture[19].

An aliquot was taken for cell count using hemocytometer under a light microscope to determine cell yield. Counts of viable cells were determined with a hemocytometer and trypan blue dye exclusion technique. Briefly, 10 µl trypan blue stock solution (0.4% w/v) was mixed with 10 µl of cell suspension, incubated for 3 minutes at room temperature, and the cells were counted in a hemocytometer. With this method, dead cells appear blue and are therefore distinguishable from viable cells.

AD-MSC culture

Isolation of MSCs from other contaminating cells was done by allowing the cells to adhere on plastic-surfaced culture dish (Nunc). Cells were seeded with a density of 40,000 cells/cm^2 in MesenCult® basal medium (Stem Cell Technology 5401), which was supplemented by MSC stimulatory supplement (Stem Cell Technology 5402 [final concentration 10%]), 100 unit/ml penicillin/0.1 mg/ml streptomycin (Sigma P4333), then kept in 37°C, 5% CO_2. After 4 days, unwanted cells (non-MSC cells and debris) were removed by two washes of medium and expanded to reach 80% confluence. In another 6-7 days, adherent cells were detached using 0.25% trypsin EDTA solution, then DMEM + 20% FBS was used to inactivate the trypsin. Detached cells with fibroblast-like morphology were cultured in a 25 cm2 flask (Nunc) for 1 week or until confluence was achieved, and the cells were used for further experiments.

AD-MSC differentiation into EPC

To differentiate the MSCs into EPCs, cells were maintained in EGM-2MV Bullet Kit medium (Cambrex CC-155; CC-4176; CC-3162), which contained 10% (v/v) heat-inactivated FBS, and 1% (v/v) penicillin–streptomycin. To determine the effect of *β*-carotene on differentiation, the culture medium was supplemented with 20 µg/mL *β*-carotene (Sigma Aldrich CAS no. 7235-40-7) compared to culture medium without *β*-carotene supplement. Culture was done in a humidified atmosphere of 95% air and 5% CO_2 for four, and seven days, and then harvested for further analysis. Mesenchymal stem cells were plated on a 24-well plate (Nunc) precoated with fibronectin (1 µg/cm^2 [Roche 10-838-039-001]) at a density of 10^4 cells/well for proliferation/viability analysis or 10^5 cells/well on a 6-well fibronectin coated plate (for EPC marker and ROS level analysis).

Cell proliferation/viability analysis

The effect of β-carotene 20 µg/mL on cell viability in EGM-2MV Bullet Kit medium was determined by cell proliferation/viability analysis. Briefly, cultured cells were dissociated using trypsin, incubated for 3 minutes in 37°C, harvested and washed using DMEM + 20% FBS followed by centrifugation at 300g, for 10 minutes. Cell pellet was resuspended, and cell count was done by trypan blue exclusion method. The experiment was done *in duplo*. Percentage of the total viable cell number was computed and noted. The mean of percentages and standard deviation of viable cells in four and seven day culture with and without β-carotene were calculated and compared.

Flowcytometry Assay of EPC markers

To confirm the effect of 20 µg/mL β-carotene in MSC differentiation, flowcytometry assays to detect EPC markers were conducted. Cultured cells were dissociated using TrypLE™Select (Gibco ME 080181), incubated for 5 minutes in 37°C, harvested and washed using DMEM + 20% FBS followed by centrifugation at 300xg, for 10 minutes. The cells were washed for 3 times using PBS + 2% FBS and centrifuge at 300xg, for 10 minutes. Cell pellet was collected and incubated in PBS + 2% FBS + FcR Blocking Reagent, at room temperature, dark condition, for 15 minutes. Afterwards, mIgG1 anti-CD34-PE/CD45-FITC (BD) 20µl, mIgG1 anti-CD133-PE (Miltenyi Biotech) 10µl, and mIgG1 anti-KDR/VEGFR-2-PE (R&D system) 15µl was added separately to cells, followed by appropriate incubation (CD 34 and CD 133 were incubated for 15 minutes, and KDR was incubated for 40 minutes, in 4 ^{0}C and in darkness). The same procedure was done for the respective isotype antibodies (20µl mIgG1-PE/mIgG1-FITC [BD] as CD34/CD45 control, 3.3 µl mIgG1-PE [BD] as CD133 control, and 9 µl mIgG1-PE [BD] as KDR control). The cells were analyzed by flowcytometry using FACSCalibur (BD Biosciences), the CD counts were computed and noted. Flowcytometry assay was done *in duplo*. The mean and standard deviations of CD counts in four and seven day cultures with and without β-carotene were calculated and compared.

Effect of β-carotene on ROS level

Quantification of intracellular ROS level was done *in duplo* by fluorescence assay using 2',7'-dichlorofluorescein diacetate (DCF-DA; Invitrogen), according to modification methods from Stolzing and Scutt (2006) and Jie *et al.* (2006) [20, 21]. After four and seven days in culture, EPCs were digested with Trypsin-EDTA and 10^4 cells were incubated with 10 µM DCF-

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DA for 30 minutes at 37 °C. After the incubation, the excess DCF-DA was washed out with PBS + KCl. Effect of *β*-carotene on ROS level was examined by incubating the cells with β-carotene (20 µg/mL) for 30 minutes, followed with H_2O_2 (final concentration 100 µM) for one hour, compared to control (without β-carotene pre-treatment). The intracellular ROS levels were measured using FACSCalibur flowcytometer (BD Biosciences). The measured ROS level values were expressed as a percentage compared to control ROS level values.

RESULTS

Effect β-carotene on cell proliferation

The effect of β-carotene treatment on cell proliferation can be seen in TABLE 1, which showed that β-carotene supplementation could increase cell proliferation both in four and seven day culture. However, higher cell number was observed after four days compared to seven days of incubation.

TABLE 1 : The effect of β-carotene on cell proliferation

Samples	Viable cell number (%)	
	4 days	7 days
Untreated	100.00± 0.00	100.00±0.00
β-Carotene (20µg/ml)	139.50±17.68	128.00±5.66

Effect of β-carotene on AD-MSCs differentiation into EPCs

TABLE 2 showed the effect of β-carotene on AD-MSCs differentiation into EPCs. Cells from culture with β-carotene supplementation showed higher EPC marker expression compared to those without supplementation. Marked KDR expression was observed after seven days of incubation with supplementation of β-carotene.

Effect of β-carotene on EPC ROS level

The effect of β-carotene on ROS intracellular level in EPCs can be seen in Figure 1, which showed that β-carotene decreased ROS intracellular level; thus decreased oxidative damage in -EPCs both in after four and seven days of incubation.

TABLE 2 : The effect of β-carotene on various EPC markers

Samples	CD34/45 (%)		CD133 (%)		VEGFR-2 (%)	
	4 days	7 days	4 days	7 days	4 days	7 days
Untreated	0.13±0.00	0.13±0.00	0.00±.00	0.00±0.00	0.00±0.00	0.00±0.00
β-carotene	0.17±0.03	0.16±0.04	0.01±0.00	3.40±0.78	0.00±0.00	2.78±0.12

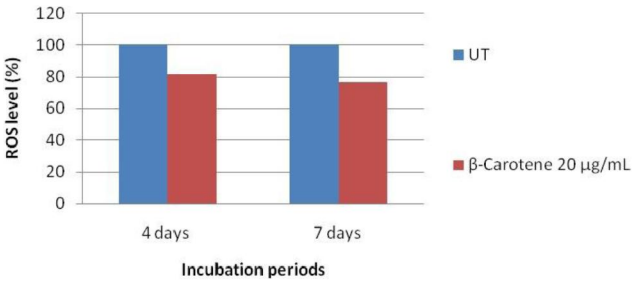

Figure 1 : Effect β-carotene supplementation on ROS level of EPCs at different incubation periods

UT= untreated

DISCUSSIONS

EGM Bullet Kit medium is a special commercial medium for endothelial cell lineage, and therefore might not support the survival and proliferation of adipose-derived stem cells, but benefited the endothelial cell lineage; a study showed that endothelial cell lineage might be present in earlier passages of adipose-derived stem cells[22]. Compared to culture without β-carotene, a marked increase in cell number was observed in culture with β-carotene supplementation after four days. The increase in proliferation might be due to the capacity of β-carotene to scavenge ROS, which production is inherent in cell growth due to oxygen consumption. Interaction of ROS with lipid produces new free radicals[7] that are involved in the production of prostaglandins, which modulate cell growth. Free radicals themselves appear to have a down regulatory effect on cell proliferation[23]. Therefore, the increase in proliferation due to β-carotene on day-4 in our study was in line with other findings, in term that β-carotene scavenge the free radicals, and thus counteract down regulation of cell proliferation[7,23].

In this study, differentiation into EPC was detected by flowcytometry using 3 markers (CD34, CD133, and

vascular endothelial growth factor receptor-2 [VEGFR-2]/KDR). VEGFR-2 is a marker to indicate endothelial characteristics, whereas CD34 and CD133 are markers to indicate cell plasticity (stem cell characteristics). Endothelial progenitor cells have originally been defined by their cell surface expression of hematopoietic marker proteins (CD133 and CD34), and endothelial marker (VEGFR-2), and their capacity to differentiate into endothelial cells *in situ,* and to induce neovascularization[4,24].

The increase in cell number was less on day-7th compared to day-4th. This result might be due to the differentiation process of a proportion of the AD-MSCs into EPCs, which was especially marked by VEGFR-2 expression (2.78%) as shown in TABLE 2, and after differentiation, the proliferation capacity was decreased. On day-7th, β-carotene supplementation caused an increase in CD 133, and insignificant increase in CD34/CD45. The increase in CD133 explained the less increase in proliferation on day-7th, as the effect of increase in CD133 as stem cell characteristics counterbalance the effect of VEGFR-2 as endothelial characteristics (differentiated cells with reduced proliferation capacity).

Intracellular ROS level measurement in this research was done by fluorescence detection of DCF-DA. 2',7'-dichlorofluorescein diacetate has been used in several studies dealing with the effect of ROS in cell culture[25-27]. 2',7'-dichlorofluorescein diacetate can cross the membrane of viable cells and is enzymatically hydrolyzed by intracellular esterases to 2',7'-dichlorofluorescin (DCFH), which is a substance without fluorescence. 2',7'- Dichlorofluorescin is rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS within the cells, and DCF remains trapped within the cell; thus can be measured to represent the intracellular ROS level[21,28].

In our study, β-carotene caused a decrease in intracellular ROS level on both day-4th and day-7th. This result might explain the mechanism of proliferation increase due to β-carotene supplementation. Mitochondria, which produce energy to drive endergonic processes of cell life, are considered as the most important cellular source of free radicals, as the main target for free radical regulatory, and as the source of signaling molecules that command cell cycle, proliferation, and apoptosis[29]. Deficiency of antioxidants is considered to be related to mitochondrial oxidative stress and dysfunction, and will damage cell cycle and trigger cells apoptosis.

Protection against oxidative stress due to ROS is accomplished by a complex defense system composed of several antioxidative enzymes that reduce the damaging effects of ROS[9]. The most vulnerable organelles to oxidative stress are the mitochondria, due to their potential for continuous production of superoxide anions. Superoxide anions are converted to hydrogen peroxide by superoxide dismutases (MnSOD), whereas hydrogen peroxide is detoxified by catalase and glutathione peroxidase (GPx-1). As the localization of MnSOD and GPx-1 is in the matrix of mitochondria, in close proximity to the production of ROS by electron transport chain, these two enzymes are believed to be the primary antioxidant defense systems in the mitochondria[9]. When the inherent defense system is inadequate, addition of ROS scavenging agents will be beneficial. Therefore, the mechanism of proliferation increase due to β-carotene supplementation in our study might be due to the ROS scavenging ability of β-carotene.

CONCLUSION

B-carotene plays a role in cell proliferation and differentiation of AD-MSCs to EPCs that might be due to decreased intracellular ROS level.

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